Study of the anti(myco)bacterial and antitumor activities of prolinate and *N*-amidocarbothiolprolinate derivatives based on fused tetrahydropyrrolo[3,4-*c*]pyrrole-1,3(2*H*,3a*H*)-dione, bearing an indole ring

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Abstract A series of prolinate and *N*-amidocarbothiolprolinate derivatives based on a fused pyrrole-3,4-dione core, bearing indole ring systems, are prepared from the corresponding amino acid and an aldehyde *via* thermal 1,3-dipolar cycloaddition of azomethine ylides and condensation with benzoylisothiocyanate. Products are fully characterized by NMR, FT- IR, MS and an X-ray crystal structure. The prepared compounds are screened for their antibacterial activity against a range of Gram-positive and Gram-negative bacteria and their antimycobacterial activity against *M. tuberculosis* H37Rv strain. In addition, two selected target compounds are evaluated for cytotoxicity, apoptosis, and anti-inflammatory effects on MCF-7 (breast

carcinoma) cell lines. The incorporation of indole ring and -C(O)NHC(S)- moiety resulted to be beneficial since the biological point of view.

Keywords Amino acids ● Cycloadditions ● Heterocycles ● MCF-7 cells ● Apoptosis ● anti(myco)bacterial

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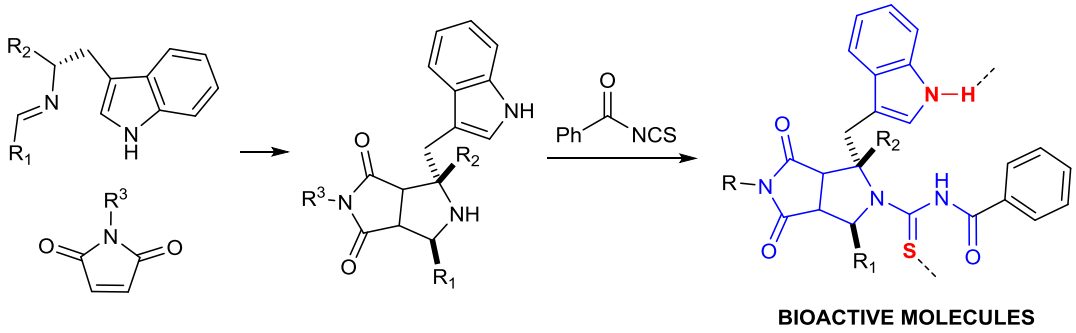
Graphical Abstract

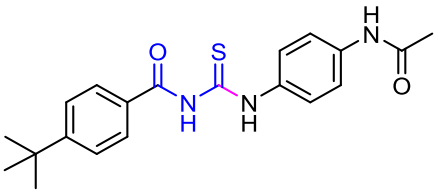


Introduction

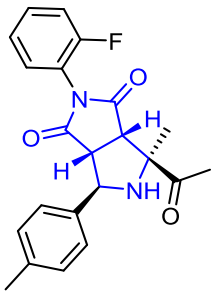
Extensive studies to synthesize compounds possessing -C(O)NHC(S)- moiety [1-4] and their metal complexes [5-7] have been focused on the preparation of variety of products with very attractive biological activity, for example, antitumoural agent [8] including a P53 activator Tenovin-1 [9] (*e.g.* **1**, Figure 1), as Nucleotide Binding Inhibitor (NBI) [10], microsomal epoxide hydrolase inhibitors [11], DNA-topoisomerase inhibitor activity [12] and some potentially important antifungal and antimicrobial activities [13-15]. Some pyrrolidine rings have been found to be bioactive in a range of biological properties [16] including antibacterial activity, virus inhibitors [17] such as HIV integrase inhibitors ZINC04885876 [18] (*e.g.* **2**, Figure 1). In addition, the presence of an indole unit is known to play an important role in medicinal chemistry increasing the anti-cancer [19,20], antimicrobial,

anti-oxidant [21-23], antitubercular, anti-inflammatory activity, for example indomethacin (*e.g.* **3**, Figure 1), and so forth [24]. So, the compounds bearing all these three pharmacophore moieties [-C(O)NHC(S)-, prolinate and indole heterocycle] are very attractive candidates to be tested in biological assais.

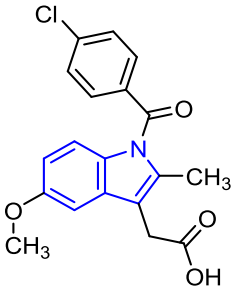
We have previously reported the synthesis and some biological evaluation of pyrrolidine, aroylaminocarbo-*N*-thioylpyrrolidine [7,25], their several metal complexes [7], thiohydantoin [26] and pyrrolidine thiazole derivatives [27]. As a continuation of our work, and according to our experience, in this article we have prepared some new selected tetrahydropyrrolo[3,4-*c*]pyrrole-1,3(2*H*,3a*H*)-dione structures incorporating -C(O)NHC(S)- moiety, prolinate and indole ring system in order to get potent anti(myco)bacterial agents and evaluate their cytotoxicity, apoptosis and anti-inflammatory effects on MCF-7 cell lines.



Tenovin-1 **1**



HIV-1 integrase inhibitor **2**



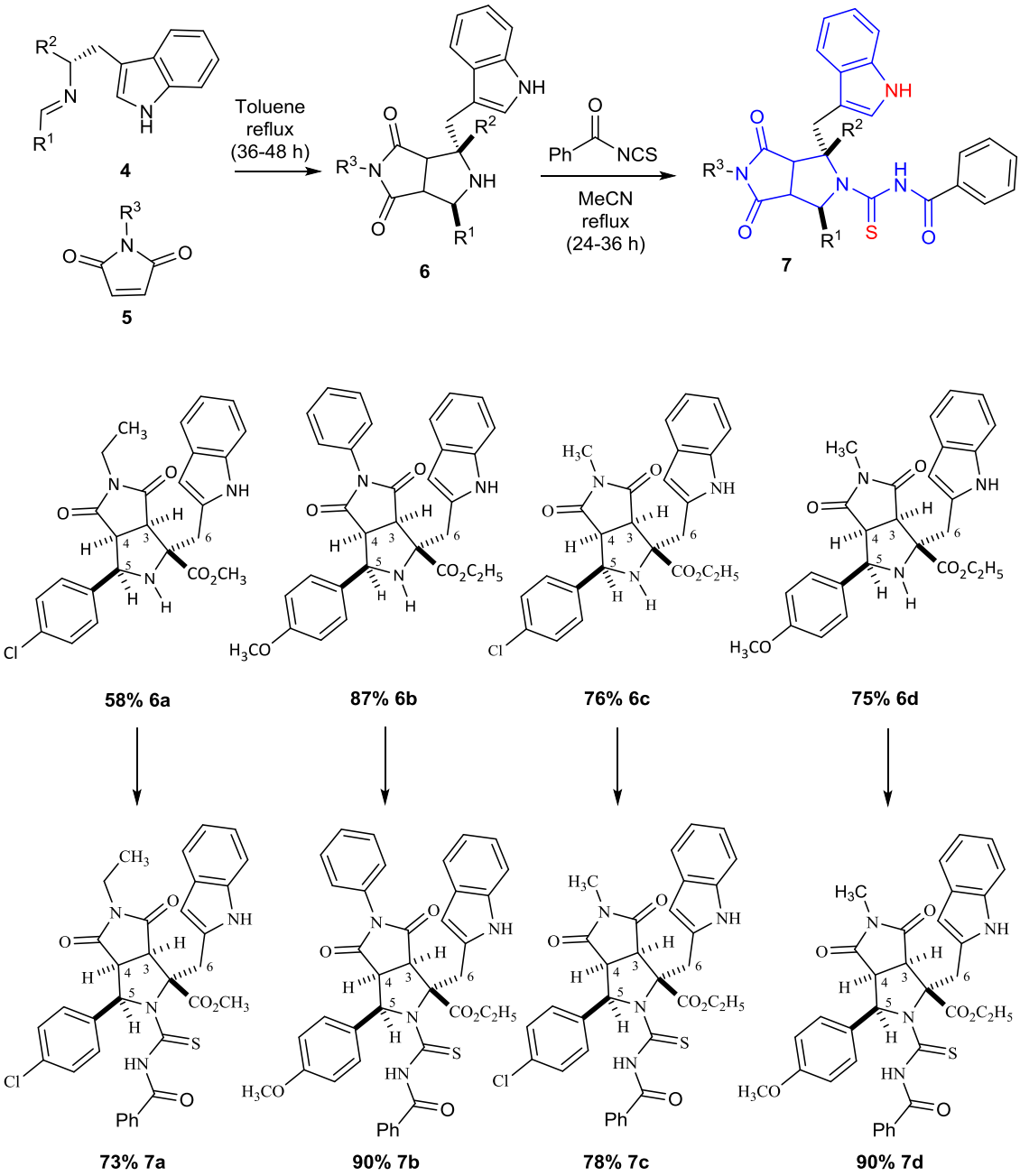
Indomethacin **3**

Figure 1. Representative examples of biologically important aroyl thiourea / pyrrolidine and indole scaffolds.

Results and Discussion

Selected novel pyrrolidines **6a-d** and aroylaminocarbo-*N*-thioylpyrrolidine **7a-d** were prepared. Prolinates **6** were obtained from the corresponding imines **4**, derived from tryptophan methyl or ethyl ester and aldehydes, *via* thermal sequence imine-azomethine ylide-1,3-dipolar cycloaddition reaction using *N*-alkyl/aryl-maleimides **5** as dipolarophiles in refluxing toluene for 36-48h (see experimental part). The prepared pyrrolidines were then condensed with benzoylisothiocyanate in dry acetonitrile at reflux for 24-36 h affording the desired functionalized *N*-amidocarbothiolpyrrolidines **7a-d** (Scheme 1), in good to excellent yields (73-90%). These reactions were performed in a larger scale (25 mmol) obtaining the same chemical yields. The relative configuration was unambiguously determined by ^{1}H NMR, ^{13}C NMR, FT- IR and MS and also by X-ray diffraction analysis of the crystalline structure **6a** (see Supporting Information).

Scheme 1


Toluene
reflux
(36-48 h)
MeCN
reflux
(24-36 h)
4
5
6
7
58% 6a
87% 6b
76% 6c
75% 6d
73% 7a
90% 7b
78% 7c
90% 7d

Antibacterial Activity

Anti-bacterial activity was investigated against *Staphylococcus aureus* (ATCC 25925), *Bacillus subtilis* (ATCC 6633), *Aeromonas hydrophila* (ATCC 95080), *Escherichia coli* (ATCC 25923) and *Acinetobacter baumanii* (ATCC 02026) which provided by the Refik Saydam Hıfzısıhha Institute. Ampicillin was used as a reference drug. The minimum inhibitory concentrations (MIC) values were determined by broth microdilution method in duplicate as recommended by the Clinical Laboratory Standards Institute [28]. Stock solutions were prepared by dissolving the compounds in DMSO and then diluting in Mueller-Hinton broth and Triptic soy broth and the test medium were prepared at concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9 and 1.9 μg/cm^3. Effects of DMSO were controlled by inoculated broth supplement at the same solutions. The observed data on the inhibited growth of bacteria at (MIC) values are given in Table 1.

The tested compounds **6a-d** and **7a-d** inhibited the growth of bacteria at (MIC) values in the range of 15.62-250 μg/cm^3. The control, ampicillin, showed activity against the tested *bacteria* with a range of 0.9-125 μg/ cm^3. The compounds **7a, 7b, 7d** were found to show the highest activity against *Aeromonas hydrophila* (ATCC 95080) in MIC values of 15.62 μg/ cm^3, and the compounds **6a, 6b, 6d** and **7b, 7d** showed better activity than ampicillin

against *Acinetobacter baumannii* (ATCC 02026) whereas the tested compounds **6a-d** and **7a-d** showed the lowest activity to other bacteria with the MIC values of 62.5- 250 µg/cm^3 as indicated in Table 1.

Table 1. The MIC values (μg/cm^3) of the target compounds against the bacteria and *M. tuberculosis* H37Rv strain.

	Staphylococcus aureus (ATCC 25925)	*Escherichia coli* (ATCC 25923)	*Acinetobacter baumannii* (ATCC 02026)	*Bacillus subtilis* (ATCC 6633)	*Aeromonas hydrophila* (ATCC 95080)	*M. tuberculosis* H37Rv
6a	125	62,5	**31,25**	125	31,25	62,5
6b	62,5	62,5	**31,25**	125	31,25	125
6c	250	250	125	250	250	62,5
6d	62,5	62,5	**31,25**	125	31,25	125
7a	62,5	62,5	62,5	125	**15,62**	**7,81**
7b	62,5	62,5	**31,25**	125	**15,62**	**7,81**
7c	250	250	125	125	250	**3,90**
7d	62,5	62,5	**31,25**	125	**15,62**	**15,62**
Ampicillin	31.25	15.62	125	0.9	31.25	
Isoniazid						0,2 and 1
Ethambutol						5 and 10

Anti-tuberculosis (TB) activity

Anti-TB activity of the compounds was investigated according to literature method [29-30] utilizing Microplate Alamar Blue assay against *M.*

tuberculosis H37Rv which provided by the Refik Saydam National Public Health Agency, National Tuberculosis Reference Laboratory, Ankara, Turkey.

The compounds **6a-d** and **7a-d** were screened and measured by means of MIC values (μg/cm^3). Ethambutol (EMB) (Sigma E4630) and Isoniazid (INH) (Sigma I3377) were used as standard reference drugs and the results with MIC values are shown in Table 1.

The tested compounds **6a-d, 7a-d** exhibited anti-tuberculosis activity in the range of 3,90 -125 μg/cm^3 suggesting that better MIC values when compared their antibacterial activity. Especially the compound **7c,** possess –Cl on the phenyl ring and –CO_2Et moiety in the pyrrolidine ring, exhibited the highest activity against *M. tuberculosis* H37Rv strain (3.90 μg/cm^3) even better activity than known reference drugs ethambutol and moderate activity when compared to isoniazid as known reference drug (Table 1). The compounds **7a**, **7b** and **7d** showed moderate activity with the MIC values of 7.81-15.62 μg/cm^3, whereas the compounds **6a-6d** showed the lowest activity with the MIC values of 62.5-125 μg/cm^3.

It is also important to note the incorporation of -C(O)NHC(S)- moiety resulted to be very beneficial in most cases.

Biological evaluation on MCF-7 (breast carcinoma) cell lines

Two selected target compounds **6a** and **7a** were chosen to test their cytotoxicity and their effects on apoptosis and inflammation on the human breast cancer MCF-7 cells.

C**ytotoxicity**

Cell proliferation experiments were carried out using the xCELLigence RTCA DP instrument, in a humidified incubator at 37°C and 5% CO_2 according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences). Cell suspension was seeded into the wells of E-plates (30,000 cells/well) [31]. Impedance value of each well was automatically monitored by the xCELLigence system or duration of 24 h and was expressed as a cell index (CI) value. At the beginning of the log phase **7a** and **6a** were added into the wells in 10, 20, 50, 100 µM concentrations. 0.01% DMSO was used as a vehicle group. Cells were grown with complete medium was used as control while 0.01% DMSO was used as a vehicle group. Three replicates of each group were used, and the experiment was ended at 100^{th} h. All data have been recorded by the supplied RTCA software (version 1.2.1).

Treatment the cells with **7a** at 10 and 20 µM caused a significant increase of cell index whereas at 50 and 100 µM caused a significant drop in the cell index (Figure 2) and IC_{50} value of **7a** was calculated as 30 µM. In addition,

treatment the cells with **6a** at 10, 20 and 50 µM caused a significant increase of cell index whereas at 100 µM caused a significant inhibition (50%) in the cell index (Figure 3).

Cell Culture

MCF-7 cells (Cat. No: 00092502 Şap Enstitüsü Ankara, TURKEY) were grown on six-well plate petri dishes containing RPMI 1640 medium supplemented with %10 fetal bovine serum (FBS), %1 L-Glutamine, %1 Penicillin-streptomycin and amphotericin-B as monolayer culture until they reached 50-60% confluence.

Biochemical analysis

In this study four groups were included. MCF-7 cells were incubated with complete medium (n=2) as control group which have no treatment. For the experimental groups, they were treated either with 0.01% DMSO (DMSO Group) or 30 µM **7a** (**7a** Group) or 100 µM **6a** (**6a** Group) in complete medium (n=2). After 24 and 48 h of treatment, cells were harvested and re-suspended in 100 µl of D-PBS for the biochemical investigations.

Apoptosis

Caspase -8 and -9 enzyme activities were evaluated as apoptotic markers [32]. For the determination of protein, MCF-7 cells were homogenized in lysis buffer by 10 min of centrifugation at 14,000 × g at 4 ◦C. Protein levels were evaluated respect to Lowry assay [33] in cytosolic extract. Caspase-8, caspase-9 and COX-2 enzyme activities were analyzed in supernatants. Caspase-8 and -9 enzyme activities were evaluated by using Caspase-8 Colorimetric Assay Kit (BioVision Research Product, Mountain View, CA, USA) and Caspase-9 Colorimetric Assay Kit (BioVision Research Product, Mountain View, CA, USA), respectively. All enzymatic activities were evaluated respect to the manufacturer's instructions. Spectrophotometric methods were based on finding out chromophore pNA after splitting from the labeled substrate. Caspase-8 and -9 enzyme activities were quantified by free *pNA* cleavage from IETD-*pNA* and LEHD-pNA respectively.

There was no statistically significant difference between the groups in caspase-8 enzyme activity which is the initiator caspase of death receptor induced (extrinsic) pathway of apoptosis (Figure 4). These results can be considered that the evaluated compounds (**6a,7a**) have no effects on this extrinsic pathway. Caspase-9 which is the apical caspase of the mitochondrial pathway and is recruited to in response to signals originating from inside the cell. There was a statistically significant increase in the **6a** 48 h group compared with the DMSO 48 h group, but in caspase activity.

Caspase-9 was also increased in **7a** 48 h group compared with the DMSO 48 h group, but it was not significant. **6a** increased apoptosis by altering caspase-9 enzyme activity, suggesting that it has potential effect on the mitochondrial (intrinsic) pathway (Figure 4).

Inflammation

COX2 levels were measured to assess inflammation in MCF-7 cells. COX2 enzyme activity was assayed respect to the manufacturer's guidance (Shanghai Sunred Biological Technology Co., Ltd. Assay kit). The results were stated in U/mg protein.

COX2 levels decreased statistically significant in **6a** 24h group compared with the control 24h groups **(**Figure 5**)**. COX1 and COX2 mediate the tissue repair process in the alimentary tract and take part in roles in tumor development at these sites [34]. COX2 is found over expressed in many cancer types [35]. Reduction of COX2 level of **6a** in MCF-7 cell lines is an important evidence for the prevention of inflammation in tumor development.

Statistical analysis

Descriptive statistics (mean ± standard deviation) were calculated in each group for all parameters. For the comparison of groups, one-way

ANOVA (ANalysis of VAriance) with *post-hoc Tukey* was used. $p<0.05$ was estimated to be statistically significant.

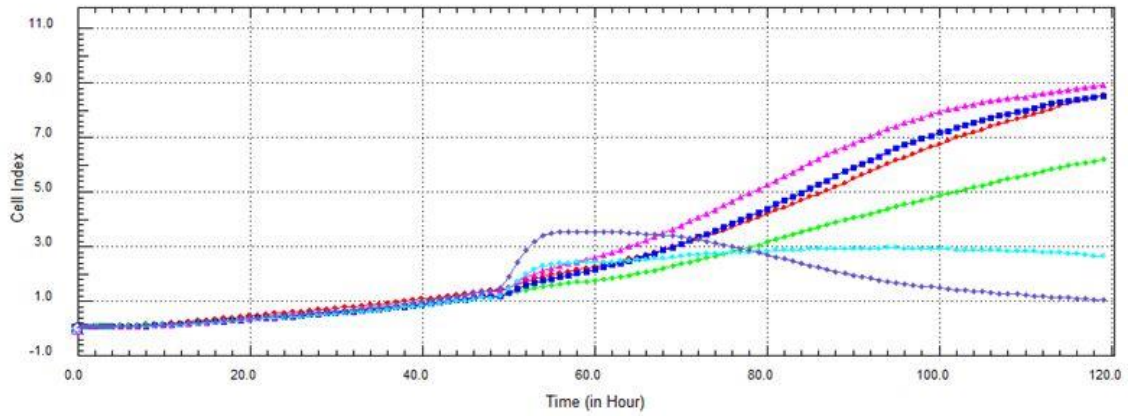


Figure 2: Red: Control, Green: DMSO group, Navy blue: **7a** at 10 µM, Pink: **7a** at 20 µM, Turquoise: **7a** at 50 µM, Purple: **7a** at 100 µM

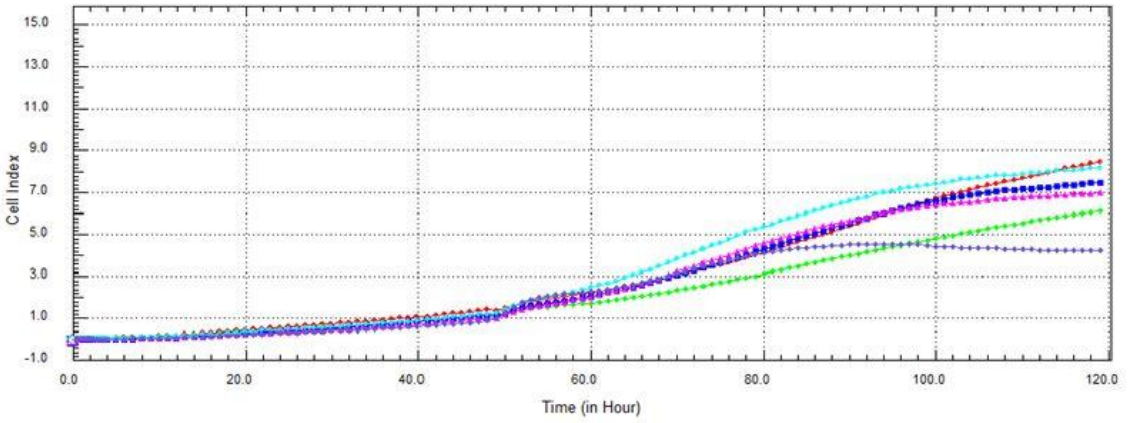


Figure 3: Red: Control, Green: DMSO group, Navy blue: **6a** at 10 µM, Pink: **6a** at 20 µM, Turquoise: **6a** at 50 µM, Purple: **6a** at 100 µM.

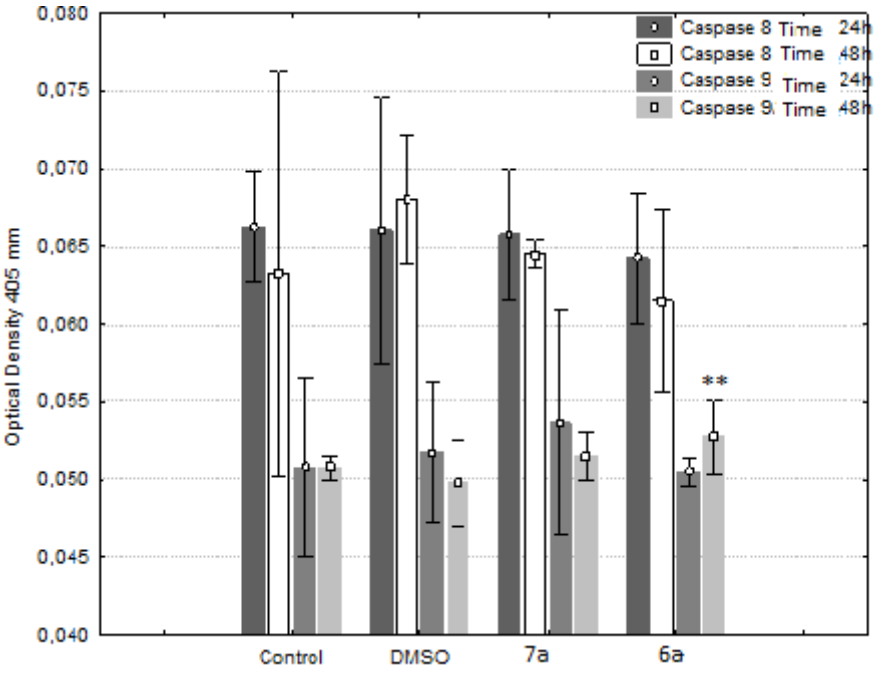


Figure 4. Effect of compounds **7a** and **6a** on Caspase 8 and on Caspase 9 on MCF-7 cell line.

** p<0.05 vs. DMSO 48 h.

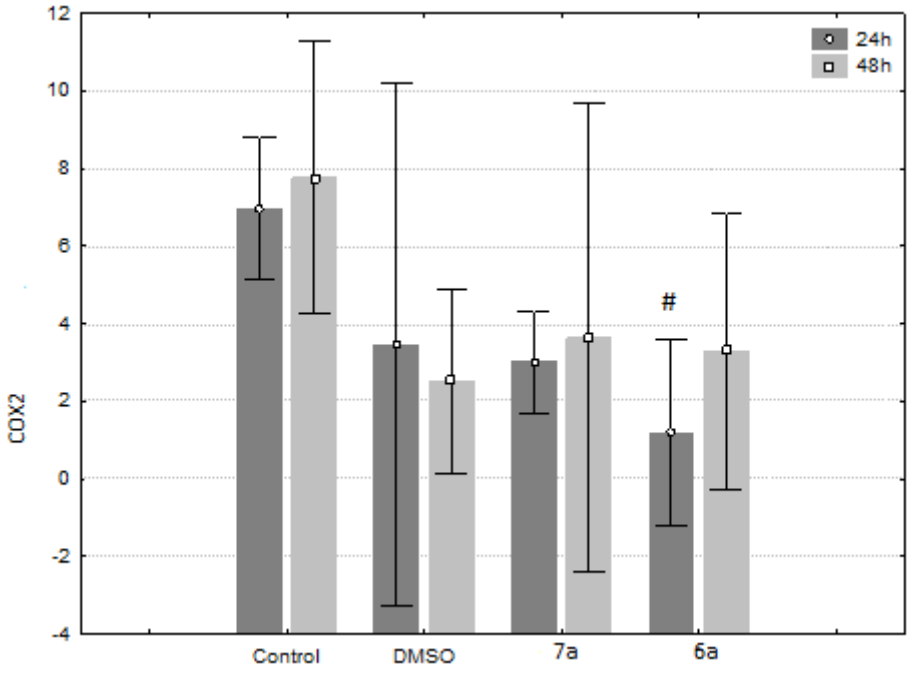
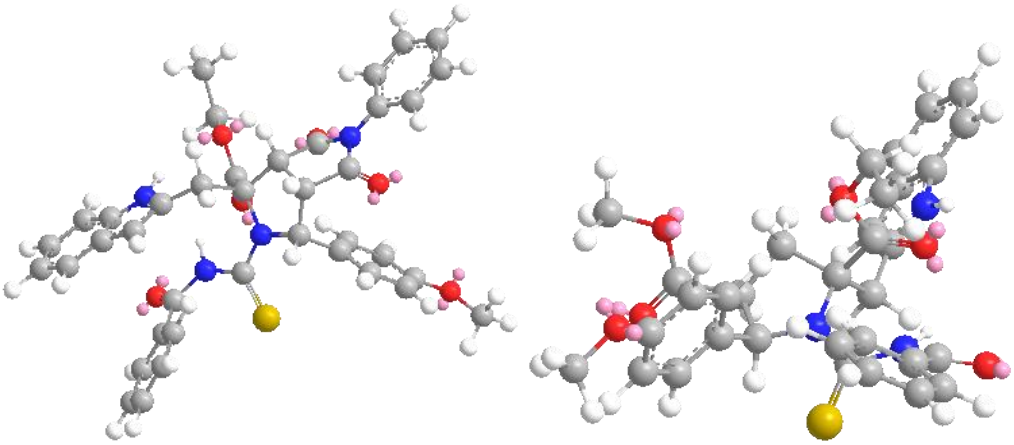


Figure 5. Effect of compounds **7a** and **6a** on COX2 on MCF-7 cell line. # $p<0.05$ vs. Control 24h.

Atomic arrangement-activity studies

Although the mode of action of these molecules with the biological targets is unknown at the moment, it is worth to furthering this work by modifying such these substrates on the aspect of structural diversity and subsequently testing of other biological activities. Structure-activity relationship (SAR) is very risky due to small amount of structured tested. However, a reasonable prediction can be attempted after analyzing the minimum energy for compounds **7b** (this work) and **8** [27] (Figure 6). The rigid core fused bicyclic entity in **7b** is very important to get the free accessible sulfur atom able to interact as nucleophile with the natural target. However, in the absence of this rigid skeleton in compound **8**, the sulfur atom is flanked by two phenyl groups avoiding a favorable approach to the living media. Two more details need to be pointed. The presence of aromatic rings can attract another π-clouds favoring specific approaches in both examples and the NH bond of the indole would promote another important hydrogen bond with a nucleophilic moiety of the biological target. In fact, a hydrogen bond can be formed between this indole-NH and the ester group in both molecules **7b** and **8** (Figure 6).



7b **8**

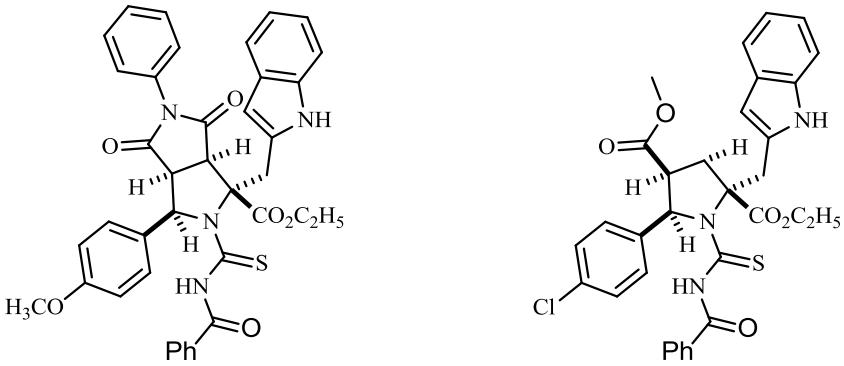


Figure 6. MM2 minimal energy calculations [36] of compounds **7b** and **8**.

Conclusion

We have described synthesis of four novel **6a-d, 7a-d** pyrrolidine and aminocarbothiol pyrrolidine derivatives based on pyrrole-3,4-dione, bearing indole ring system in good yields. The prepared compounds were screened for their anti(myco)bacterial activity against a range of bacteria and *M. tuberculosis* H37Rv strain. The screened compounds showed moderate

antibacterial activity against various bacteria strains whereas showed better anti-TB activity against *M. tuberculosis* H37Rv strain. In general, better bactericide activity was observed than those previously reported by our group. The obtained results displayed that these target compounds can be considered as potential anti(myco)bacterial activity agent, especially anti tuberculosis agent (*e.g.* **7a, 7b** and **7c***).* The compound **7c** exhibited higher activity against *M. tuberculosis* H37Rv strain in 3.90 µg/ cm^3 values and show even better activity than known reference drugs ethambutol and moderate activity when compared to isoniazid as known reference drugs. Moreover, two selected target compounds **6a** and **7a** were evaluated for cytotoxicity, apoptosis and anti-inflammatory effects on MCF-7 cell lines. In addition, the tested compounds **6a** and **7a** reflect moderate activity against some of the used tumor cells which can be consider as potential bioactive to possible anti-carcinogenic properties on MCF-7 cell lines. It is also important to note that the incorporation of indole ring and -C(O)NHC(S)- moiety resulted to be crucial for the increment of activity since the biological point of view, so they can consider promising candidates for antitumor agents. As tentative proposal the sulfur atom can play a crucial role in the effective interaction with the living target in order to get the experimentally observed inhibition of the cell/bacteria.

Experimental

All chemicals were purchased from Merck or Sigma-Aldrich and used without further purification. Melting points were determined on a Stuart SMP3 hot stage apparatus and are uncorrected. The structurally most important peaks of the IR spectra (Perkin-Elmer FT-IR using horizontal ATR) are listed and wave numbers are given in cm^{-1}. Nuclear magnetic resonance spectra were determined on a Bruker Ultra Shield Plus Biospin GmbH 400 MHz spectrometer. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane as internal standard. Spectra were determined in deuterated chloroform or DMSO-d_6 as stated. The following abbreviations are used; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br s = broad singlet. Flash column chromatography was performed using silica gel 60. Mass spectra were recorded on an Agilent LC/MSD, Agilent 6460 Triple Quad LC/MS/MS mass spectrometer. The X-ray crystal structure data were collected on Bruker APEX-II CCD model X-ray diffractometer. Flash column chromatography was performed using silica gel 60 (230-400 mesh). High-resolution mass spectra (HRMS) were measured in a Finnigan MAT 95S using ESI.

*General Procedure for the synthesis of bicyclic pyrrolidines **6***

The pyrrolidine derivatives **6a-d** prepared by adaption of literature procedures [4,7,15,27]. Thus, aldehydes (1,1 mmol) in 10 cm^3 were added to L-tryptophan methyl/ethyl esters (1mmol) in 20 cm^3 dry DCM to form corresponding imines **4**. Then, N-maleimides (1mmol) in dry toluene were added to imine solution (1 mmol). The reaction mixture was stirred under reflux for 36-48 h. After completion of the reaction by monitoring TLC, the reaction mixture was evaporated and crystallized from ether-hexane. R_f values for each compounds **6a-d** were calculated in (Et_2O: hexane, 2:1).

(1S,3R,3aS,6aR)-methyl 1-((1H-indol-2-yl)methyl)-3-(4-chlorophenyl)-5-ethyl-4,6-dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (**6a,** $C_{25}H_{24}ClN_3O_4$)

Crystallized from Et_2O: hexane as colorless prisms. Yield 58 %: m.p.: 237-239 ºC. R_f: 0.20, ^{1}H NMR (400 MHz, DMSO): δ = 11.00 (br s, 1H, NH), 7.57-6.95 (m, 9H, Ar-H), 5.02 (dd, 1H, *J*= 4.68 Hz, *J*= 9.4 Hz, 5-H), 3.72 (dd, 1H, *J*= 8.38 Hz, *J*= 7.52 Hz, 4-H), 3.68 (s, 3H, OCH_3), 3.60 (dd, 1H, *J*= 7.46 Hz, *J*= 1.38 Hz, 3-H), 3.43 (d, 1H, *J*= 14.6 Hz, 6-H), 3.30 (d, 1H, *J*= 14.56 Hz, 6- H'), 3.25-3.13 (m, 2H, CH_2CH_3), 2.44 (d, 1H, *J*= 3.52 Hz, N-H), 0.91 (t, 3H, *J*= 7.16 Hz, CH_2CH_3) ppm; ^{13}C NMR (100 MHz, DMSO): δ= 175.79 (C=O), 174.6 (C=O), 171.6 (C=O), 138.2, 135.9, 131.8, 129.2 (2 C), 127.8 (2 C), 127.6, 124.4, 121.0, 118.6, 118.0, 111.5, 107.9, 70.2, 58.8,

53.4, 51.5, 48.5, 32.9, 30.3, 12.7 ppm; IR $\bar{\nu}$ = 3339, 2981, 2944, 2840, 1774 (C=O), 1739 (C=O), 1683 (C=O), 744 cm^{-1}, *m/z* = 466.3 ($[M+H]^+$, 100): 468.3 ($[M+H]^+$, 35); HRMS (ES): $C_{25}H_{24}ClN_3O_4$ calc. ($[M+H]^+$ 465.1455, found 465.1449.

(1S,3R,3aS,6aR)-ethyl 1-((1H-indol-2-yl)methyl)-3-(4-methoxyphenyl)-4,6-dioxo-5-phenyloctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (**6b**, $C_{31}H_{29}N_3O_5$)

Crystallized from Et_2O: hexane as colorless prisms. Yield 87 %: m.p.: 268-270 ºC (decomp.), R_f: 0.24, ^1H NMR (400 MHz, DMSO): δ = 11.01 (bs, 1H, NH), 7.60-6.89 (m, 14H, Ar-H), 5.11 (dd, 1H, *J* = 9.38 Hz, 4.8 Hz, 5-H), 4.13-4.04 (m, 2H, CH_2CH_3), 3.88 (dd,1H, *J* = 9.34 Hz, 7.68 Hz, 4-H), 3.77 (dd, 1H, *J* = 7.56 Hz, 1,44 Hz, 3-H), 3.74 (s. 3H, OCH_3), 3.44 (d, 1H, *J* = 14.56 Hz, 6-H), 3.34 (d, 1H, *J* = 14.50 Hz, 6-H'), 2.44 (d, 1H, *J* = 4.68 Hz, N-H), 1.25 (t, 3H, *J* = 7.12 Hz, CH_2CH_3) ppm; ^{13}C NMR (100 MHz, DMSO): δ= 175.4 (C=O), 174.2 (C=O), 171.3 (C=O), 158.7, 136.9, 132.2, 130.8, 128.8 (2 C), 128.5 (2 C), 128.2, 127.7, 126.7 (2 C), 124.3, 121.0, 120.2, 118.5, 118.1, 113.3, 111.5, 108.0, 70.4, 60.4, 59.5, 55.0, 54.1, 49.1, 30.2, 13.9 ppm ; IR $\bar{\nu}$ = 3356, 3067, 2995, 1772, 1708 cm^{-1}, MS (ESI) *m/z* = 524.3 ($[M+H]^+$, 100).

(*1S,3R,3aS,6aR)-ethyl 1-((1H-indol-2-yl)methyl)-3-(4-chlorophenyl)-5-methyl-4,6-dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylate* (**6c,** $C_{25}H_{24}ClN_3O_4$)

Crystallized from Et_2O: hexane as colorless prisms. Yield 76 %; m.p.: 213-215 ºC (decomp.), R_f: 0.20, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.25 (bs, 1H, NH), 7.56 (d, 1H, *J* = 7.88 Hz, Ar-H), 7.25-6.96 (m, 8H, Ar-H), 4.85 (dd, 1H, *J* = 9.1 Hz, 3.60 Hz, 5-H), 4.33-4.17 (m, 2H, C*H*$_2$CH$_3$), 3.61 (d, 1H, *J* = 14.68 Hz, 6-H), 3.55 (dd, 1H, *J* = 9.0 Hz, 7.66 Hz, 4-H), 3.44 (d, 1H, *J* = 7.48 Hz, 3-H), 3.24 (d, 1H, *J* = 14.64 Hz, 6-H'), 2.79 (s, 3H, NCH_3), 2.50 (d, 1H, *J* = 3.16 Hz, N-H), 1.34 (t, 3H, *J* = 7.16 Hz, CH_2C*H*$_3$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ= 175.7 (C=O), 174.7 (C=O), 171.6 (C=O), 137.8, 136.4, 135.9, 130.0, 128.6 (3C), 126.1, 123.2, 122.4, 119.9, 118.2, 111.5, 109.2, 71.1, 61.7, 60.6, 54.0, 49.5, 24.9, 21.4, 14.1 ppm; IR: $\bar{\nu}$ = 3324, 2959, 1777, 1725, 1698, 1430 1282, 1198, 1091, 1012,785, 740 cm^{-1} , MS (ESI) *m/z* = 466.2 ([M+H]$^+$, 100): 468,2 ([M+H]$^+$, 35); HRMS (ES): $C_{25}H_{24}ClN_3O_4$ calc. ([M+H]$^+$ 465.1455, found 465.1449.

1S,3R,3aS,6aR)-ethyl 1-((1H-indol-2-yl)methyl)-3-(4-methoxyphenyl)-5-methyl-4,6-dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (**6d**, $C_{26}H_{27}N_3O_5$)

Crystallized from Et_2O: hexane as colorless prisms. Yield 75 %; m.p.: 185-187 ºC, R_f: 0.16, ^{1}H NMR (400 MHz, DMSO): δ = 8.19 (s, 1H, NH), 7.59 (d, 1H, *J* = 7.88 Hz, Ar-H), 7.25-6.80 (m, 8H, Ar-H), 4.85 (dd, 1H, *J* = 9.06 Hz, 4.76 Hz, 5-H), 4.33-4.16 (m, 2H,CH_2CH_3), 3.75 (s, 3H, OCH_3), 3.59 (d, 1H, *J* = 14.56 Hz, 6-H), 3.53 (dd, 1H, *J* = 9.00 Hz, 7.52 Hz, 4-H), 3.45 (d, 1H, *J* = 7.44 Hz, 3-H), 3.26 (d, 1H, *J* = 14.64 Hz, 6-H'), 2.80 (s, 3H, NCH_3), 2.55 (d, 1H, *J* = 4.52 Hz, N-H), 1.32 (t, 3H, *J* = 7.16 Hz, CH_2CH_3) ppm; ^{13}C NMR (100 MHz, DMSO): δ= 175.9 (C=O), 175.1 (C=O), 171.7 (C=O), 159.5, 136.9, 129.8, 128.3 (2 C), 127.9, 123.2, 122.2, 119.8, 118.3, 113.8, (2 C), 111.3, 109.5, 71.0, 61.6, 61.0, 55.2, 54.3, 49.8, 30.8, 24.9, 14.1 ppm; IR $\bar{\nu}$ = 3414, 3342, 2981, 1780, 1727 cm^{-1}, MS (ESI) *m/z* = 462.3 ([M+H]$^+$, 100).

General Procedure for the synthesis of bicyclic benzoylaminocarbo-N-thiol-pyrrolidines ***7***

Bicyclic benzoylaminocarbo-*N*-thioylpyrrolidines **7a-d** were prepared by adaption of literature procedures [4,7,15,27]. Thus, to a stirred solution of pyrrolidine (1.2 mmol) in 25 cm^3 dry acetonitrile was added a solution of benzoyl isothiocyanate (1.22 mmol) in 15 cm^3 of dry acetonitrile in dropwise. The resulting mixture was stirred at reflux for appropriate time (24-36 h) monitoring by TLC. After completion of the reaction, the solvent

removed and purified by flash chromatography. *Rf* values for compounds **7a-d** were calculated in Et_2O: hexane 2:1.

(1S,3R)-methyl 1-((1H-indol-3-yl)methyl)-2-(benzoylcarbamothioyl)-3-(4-chlorophenyl)-5-ethyl-4,6-dioxo-octahydropyrrolo[3,4-c]pyrrole-1 carboxylate (**7a,** $C_{33}H_{29}ClN_4O_5S$)

Reaction time 24 h. The product crystallized from Et_2O: hexane as pale-yellow prisms as a 1:3 rotamer mixture. Yield 73 %; m.p.: 145-147 ºC; R_f: 0.25, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.55 (br s, 1H, NH, major rotamer), 8.46 (br s, 1H, NH minor rotamer), 8.19-7.00 (m, 30H, Ar-H, NH major and minor), 5.63 (d, 1H, *J* = 11.52 Hz, 5-H minor), 5.43 (d, 1H, *J* = 11.2 Hz, 5-H major), 4.81 (d, 2H, *J*= 15.44 Hz NC*H*$_2$$CH_3$ major), 4.42 (d, 2H, *J*= 15.04 Hz NC*H*$_2$$CH_3$ minor), 3.96 (s, 3H, OC*H*$_3$ major), 3.90 (s, 3H, OC*H*$_3$ minor), 3.20-2.99 (m, 4H, 6-H, 6-H' major and minor), 2.77- 2.44 (m, 2H, 3-H major and minor), 1.41-1.19 (m, 5H, 4-H major and minor, CH_2C*H*$_3$ minor), 0.68 (t, 3H, *J*= 7.2 Hz, CH_2C*H*$_3$ major) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ= 187.3 (C=S), 178.8 (C=O), 173.8 (C=O), 173.1 (C=O), 170.2 (C=O), 135.9, 135.0, 133.9, 33.6, 133.3, 133.1, 129.2 (2 C), 128.8, 128.7 (2 C), 127.8, 127.6 (2 C), 124.3, 122.9 , 121.7, 120.7, 117.9, 69.0, 68.3, 53.1, 49.4, 47.8, 34.0, 33.9, 12.5, 12.3 ppm; IR: $\bar{\nu}$ = 3374, 2981, 2946, 2925, 1782 (C=O), 1740 (C=O), 1702 (C=O), 1228, 710 cm^{-1}, MS (ESI): *m/z* = 627.3 ([M-H$^+$],

100), 629.3 ([M-H^+], 45), HRMS (ES): $C_{33}H_{29}ClN_4O_5S$ calc. ([M+H]$^+$ 628.1547, found 628.1546.

1S,3R,3aS,6aR)-ethyl 1-((1H-indol-2-yl)methyl)-2-(benzoylcarbamothioyl)-3-(4-methoxyphenyl)-4,6-dioxo-5-phenyloctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (**7b,** $C_{39}H_{34}N_4O_6S$)

Reaction time 24 h. The product crystallized from Et_2O: hexane as pale-yellow prisms as a 1:1 rotamer mixture. Yield 90 %; m.p.: 170-172 ºC; R_f: 0.20, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.56 (br s, 1H, NH major rotamer), 8.49 (br s, 1H, NH minor rotamer), 8.22-6.51 (m, 40H, Ar-H, NH major and minor), 5.57 (d, 1H, *J* = 11.00 Hz, 5-H minor), 5.53 (d, 1H, *J* = 11.32 Hz, 5-H major), 4.98 (d, 1H, *J* = 15.4 Hz, 6-H minor), 4.48 (d, 1H, *J* = 15.24 Hz, 6- H' minor), 4.43-4.18 (m, 4H, CH_2CH_3 major and minor), 3.94 (d, 1H, *J* = 14.96 Hz, 6-H major), 3.91-3.83 (m, 2H, 4-H major and minor) 3.86 (d, 1H, *J* = 14.96 Hz, 6- H' major), 3.74 (s, 3H, OCH_3 minor), 3.73 (s, 3H, OCH_3 major), 2.82 (dd, 1H, *J*=10.44 Hz, 10.32 Hz, 3-H major), 2.55 (dd, 1H, *J*= 10.9 Hz, 9.04 Hz, 3-H minor), 1.43 (t, 3H, *J*= 6.96 Hz, CH_2CH_3 major), 1.30 (t, 3H, *J*= 7.12 Hz, CH_2CH_3 minor) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ= 187.0 (C=S major), 173.0 (C=S minor), 172.9 (C=O major), 172.6 (C=O major), 172.3 (C=O minor), 171.9 (C=O minor), 169.6 (C=O major), 167.8 (C=O minor), 164.0 (C=O minor), 159.1 (C=O major), 135.9 (2 C major),

133.7 (C major), 133.7 (C minor), 133.1 (C minor), 132.9 (C major), 132.7 (C major), 131.7 (C major), 131.0 (C minor), 130.9 (C major), 130.2 (2 C minor), 129.2 (2 C minor), 129.0 (4 C major), 128.9 (3 C minor), 128,6 (4 C major), 128,5 (2 C minor), 127.8 (C minor), 127.7 (C minor), 127.6 (2 C minor), 127.5 (C minor), 126.7 (C minor), 125.9 (5 C major), 125.7 (C minor), 124.3 (C major), 122.8 (C major), 122.5 (C minor), 120.6 (C major), 120.4 (C minor), 118,1 (C major), 117.9 (C minor), 113.9 (C minor), 113.7 (C major), 111.9 (C major), 111.8 (C minor), 108.9 (C major), 108.5 (C minor), 76.1 (minor), 69.1 (C major), 68.6 (C minor), 62,1 (C major), 55.4 (C major), 55.2 (2 C minor), 54.3 (C minor), 52.8 (C major), 49.5 (C minor), 48.2 (C major), 33.0 (C major), 29.7 (C minor), 28.2 (C minor), 14.0 (2 C major) ppm; IR: $\overline{\nu}$ = 3440, 2990, 1731, 1711, 1245, 1213 cm^{-1}, MS (ESI) *m/z* = 687.3 ([M-H+], 100).

(1S,3R,3aS,6aR)-ethyl 1-((1H-indol-3-yl)methyl)-2-(benzoylcarbamothioyl)-3-(4-chlorophenyl)-5-methyl-4,6-dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (**7c**, $C_{33}H_{29}ClN_4O_5S$)

Reaction time 24 h. The product crystallized from Et_2O: hexane as pale-yellow prisms as a 1:1 rotamer mixture. Yield 78 %; m.p.: 128-130 ºC; R_f: 0.22, ^1H NMR (400 MHz, $CDCl_3$): δ = 8.61 (br s, 1H, NH, minor rotamer), 8.55 (br s, 1H, NH major rotamer), 8.20 (s, 1H, NH minor), 8.18 (s, 1H, NH

major), 7.79-7.02 (m, 28H, Ar-H, major and minor), 5.60 (d, 1H, *J* = 11.40, 5-H minor), 5.40 (d, 1H, *J* = 11.08, 5-H major),4.59-4.20 (m, 8H, C*H*$_2$CH$_3$, 4-H major and minor), 3.94 (d, 1H, *J* = 15.52, 6-H major), 3.87 (d, 1H, *J* = 14.36, 6-H' minor), 3.73-3.68 (m, 2H, 3-H major and minor), 2.57 (s, 3H, NCH$_3$ minor), 2.45 (s, 3H, NCH$_3$ major),1.48-1.33 (m, 6H, CH$_2$C*H*$_3$ major and minor) ppm; ^{13}C NMR (100 MHz, CDCl$_3$): δ= 187.2 (C=S major),179.7 (C=S minor), 174.1 (C=O minor), 173.8 (C=O major), 173.3 (C=O major), 173.0 (C=O minor), 172.1 (C=O minor), 169.6 (C=O major), 169.4 (C=O minor), 164.0 (C=O major), 135.9 (C major), 135.8 (C minor), 135.4 (C major), 134.9 (C minor), 134.6 (C minor), 133.9 (C major), 133.7 (C major), 133.3 (C minor), 133.1 (C major), 133.0 (C minor), 132.3 (C minor), 132.1 (C major), 131.9. (C major), 129.23 (2 C minor), 129.0 (C minor), 128.7 (2 C major), 128.6 (C major), 128.5 (C minor), 128.3 (C major), 128.0 (C minor), 127.8 (C minor), 127.6 (C major), 127.4 (2 C minor), 126.2 (2 C major), 126.0 (C minor), 124.4 (C major), 122.8 (C major), 122.4 (C minor), 120.6 (C major), 120.3 (C minor), 117.9 (C minor), 117.8 (C major), 111.9 (C major), 111.8 (C minor), 108.7 (C major), 108.1 (C minor), 76.1 (C major), 68.8 (C minor), 68.3 (C minor), 62.5 (C major), 54.1 (C major), 52.4 (C minor), 49.5 (C minor), 48.0 (C major), 36.7 (C minor), 32.9 (C major), 27.70 (C minor), 24.7 (C major), 24.5 (2 C minor), 14.0 (2 C major) ppm; IR: $\bar{\nu}$ =3364, 3055, 2975, 2937, 1785, 1707, 1599, 1563, 1489, 1377, 1232,

1090, 745, 711 cm^{-1}, MS (ESI): *m/z* = 627.2 ([M-H^+], 100), 629.2 ([M-H^+], 42), HRMS (ES): $C_{33}H_{29}ClN_4O_5S$ calc. ([M+H]$^+$ 628.1547, found 628.1544.

(1S,3R,3aS,6aR)-ethyl1-((1H-indol-2-yl)methyl)-2-(benzoylcarbamothioyl)-3-(4-methoxyphenyl)-5-methyl-4,6 dioxooctahydropyrrolo[3,4-c]pyrrole-1-carboxylate (**7d,** $C_{34}H_{32}N_4O_5S$)

Reaction time 24 h. The product crystallized from Et_2O: hexane as pale-yellow prisms as a 1:1 rotamer mixture. Yield 90 %; m.p.: 198-200 ºC; R_f: 0.14, ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.52 (br s, 1H, NH, major rotamer), 8.47 (br s, 1H, NH minor rotamer), 8.20-7.04 (m, 30H, Ar-H, NH major and minor), 4.92 (d, 1H, *J* = 15.32 Hz, 5-H minor), 4.86 (d, 1H, *J* = 9.16 Hz, 5-H major), 4.44-4.19 (m, 6H, CH_2CH_3, 4-H major and minor), 3.96-3.79 (m, 4H, 6-H, 6-H' major and minor), 4.00 (d, 1H, *J*=15.16 Hz, 3-H minor), 3.86 (d, 1H, *J*= 4.56 Hz, 3-H major), 3.76 (s, 3H, OC*H*$_3$ minor), 3.73 (s, 3H, OC*H*$_3$ minor), 2.81 (s, 3H, NCH_3 minor), 2.45 (s, 3H, NCH_3 major), 1.47 (t, 3H, *J*= 7.12 Hz, CH_2CH_3 major), 1.33 (t, 3H, *J*= 7.12 Hz, CH_2CH_3 minor) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ= 186.9 (C=S minor), 174.1 (C=S major), 173.9 (C=O minor), 173.5 (C=O major), 173.2 (C=O minor), 171.8 (C=O major), 169.7 (C=O major), 164.0 (C=O minor), 159.9 (C=O minor), 159.0 (C=O major), 136.9 (3mC major), 133.7 (2 C minor), 133.2 (2 C minor), 132.9 (C major), 132.7 (C minor), 129.2 (3 C major), 128.7 (4 C minor), 128.6. (C

major), 127.8 (3 C minor), 127.6 (5 C major), 125.7 (C minor), 124.3 (2 C major), 122.7 (2 C minor), 122.4 (C major), 120.6 (2 C minor), 120.4 (C major), 118.0 (C minor), 117.9 (C major), 111.8 (C major), 111.7 (C minor), 108.9 (C major), 108.5 (C minor), 68.9 (C minor), 68.6 (C major), 62.3 (2 C minor), 55.3 (C major), 55.1 (C major), 54.1 (C minor), 52.7 (C major), 49.6 (C minor), 48.2 (C minor), 32.9 (C major), 28.1 (C minor), 24.6 (C major), 24.4 (2 C minor), 14.0 (3 C major) ppm; IR: $\bar{\nu}$= 3346, 2980, 1734, 1706, 1246, 1210 cm^{-1} , MS (ESI) *m/z* = 625.3 ([M-H+], 100).

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36. These calculations were run with ChemBio Draw program using the MM2 package at a basic level.

Figure Captions

Figure 1. Representative examples of biologically important aroyl thiourea / pyrrolidine and indole scaffolds.

Figure 2: Red: Control, Green: DMSO group, Navy blue: **7a** at 10 µM, Pink: **7a** at 20 µM, Turquoise: **7a** at 50 µM, Purple: **7a** at 100 µM

Figure 3: Red: Control, Green: DMSO group, Navy blue: **6a** at 10 µM, Pink: **6a** at 20 µM, Turquoise: **6a** at 50 µM, Purple: **6a** at 100 µM.

Figure 4. Effect of compounds **7a** and **6a** on Caspase 8 and on Caspase 9 on MCF-7 cell line.

** p<0.05 vs. DMSO 48 h.

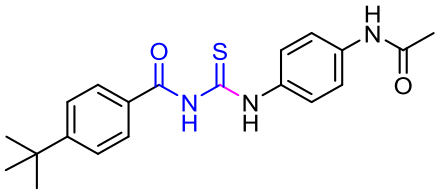
Figure 5. Effect of compounds **7a** and **6a** on COX2 on MCF-7 cell line. # p<0.05 vs. Control 24h.

Figure 6. MM2 minimal energy calculations [36] of compounds **7b** and **8**.

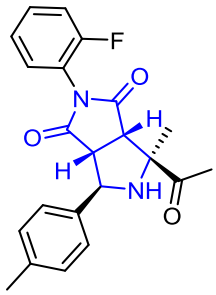
Table 1. The MIC values (μg/cm^3) of the target compounds against the bacteria and *M. tuberculosis* H37Rv strain.

	Staphylococcus aureus (ATCC 25925)	*Escherichia coli* (ATCC 25923)	*Acinetobacter baumannii* (ATCC 02026)	*Bacillus subtilis* (ATCC 6633)	*Aeromonas hydrophila* (ATCC 95080)	*M. tuberculosis* H37Rv
6a	125	62,5	**31,25**	125	31,25	62,5
6b	62,5	62,5	**31,25**	125	31,25	125
6c	250	250	125	250	250	62,5
6d	62,5	62,5	**31,25**	125	31,25	125
7a	62,5	62,5	62,5	125	**15,62**	**7,81**
7b	62,5	62,5	**31,25**	125	**15,62**	**7,81**
7c	250	250	125	125	250	**3,90**
7d	62,5	62,5	**31,25**	125	**15,62**	**15,62**
Ampicillin	31.25	15.62	125	0.9	31.25	
Isoniazid						0,2 and 1
Ethambutol						5 and 10

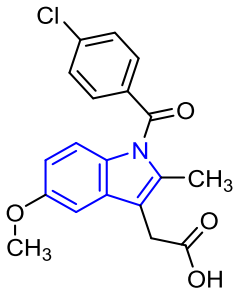
Figure 1



Tenoviv **1**



HIV-1 integrase inhibitor **2**



Indomethacin **3**

Figure 2

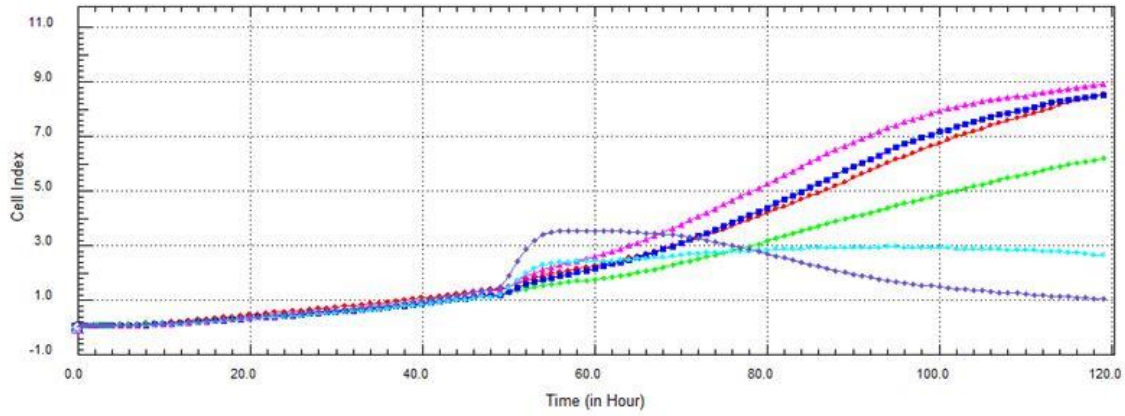


Figure 3

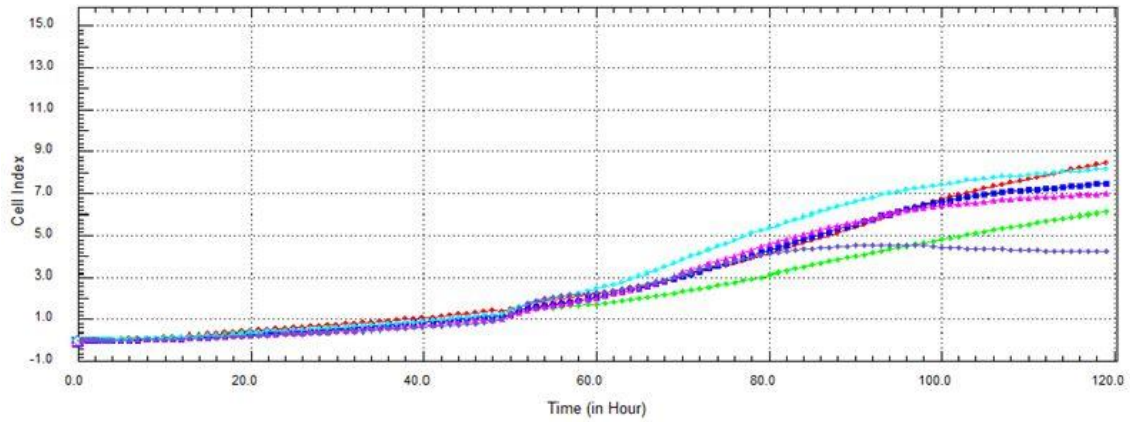


Figure 4

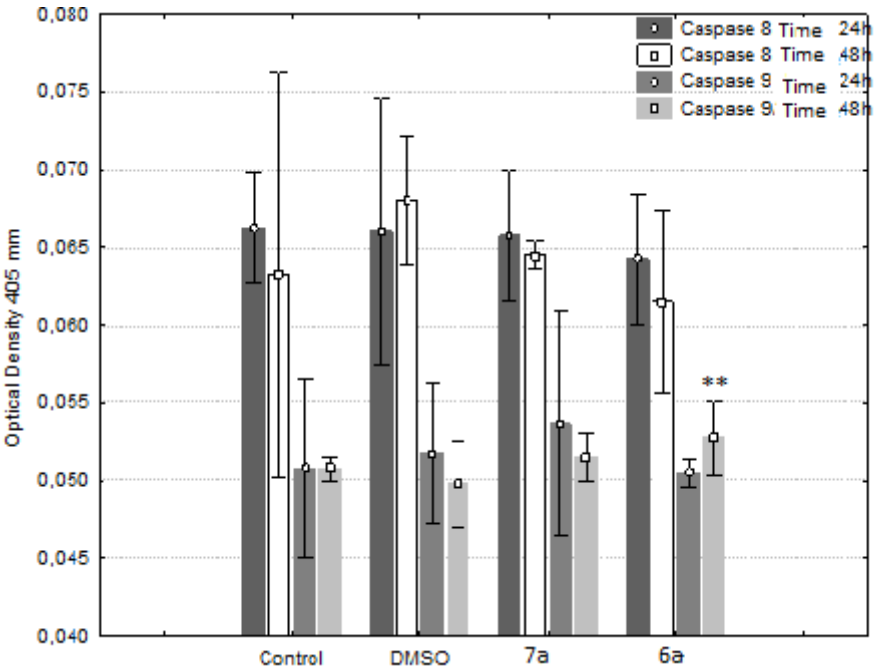
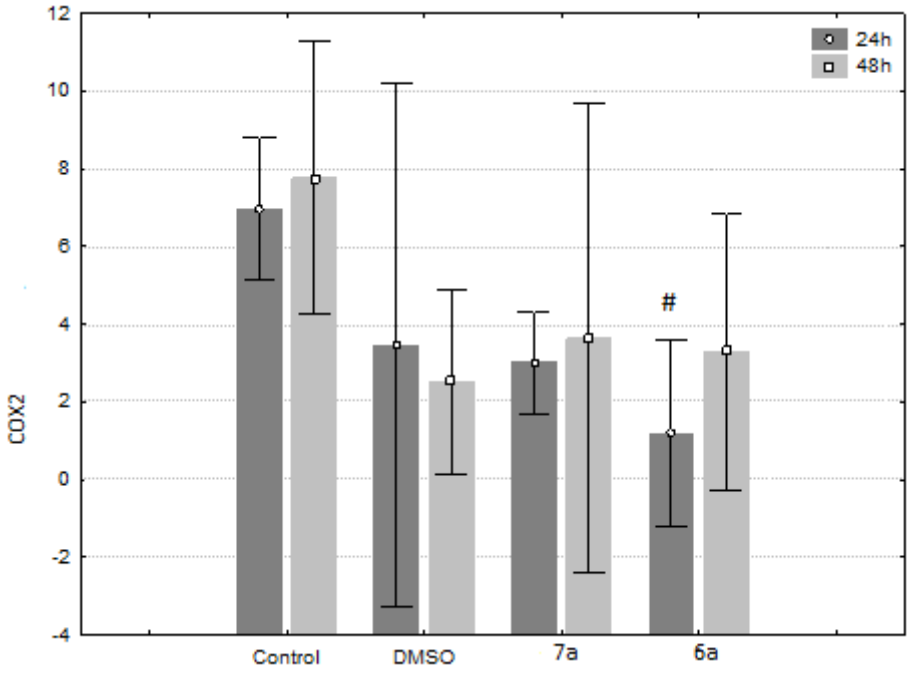
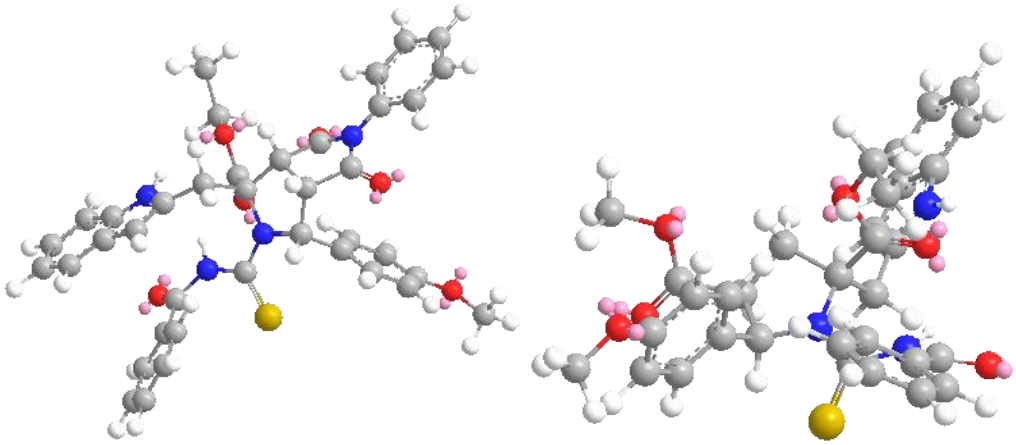


Figure 5





7b **8**

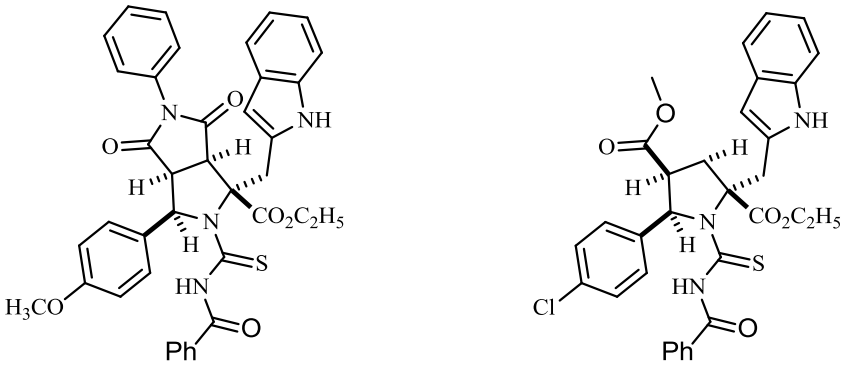
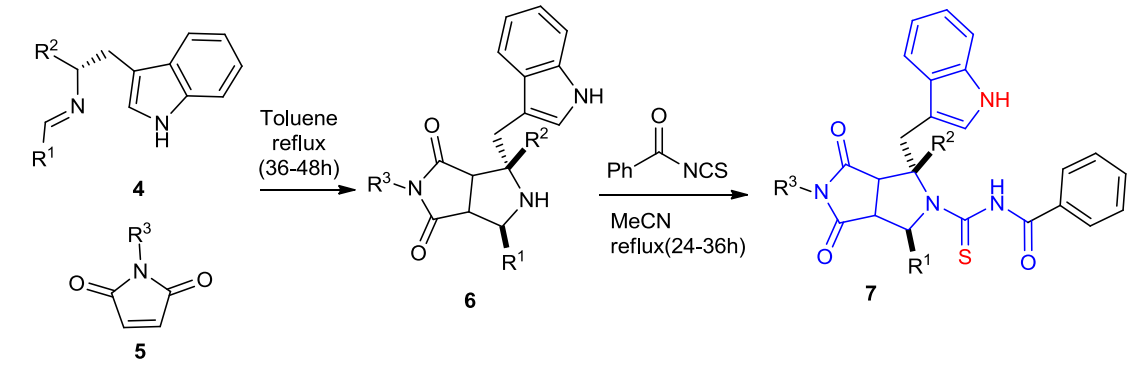
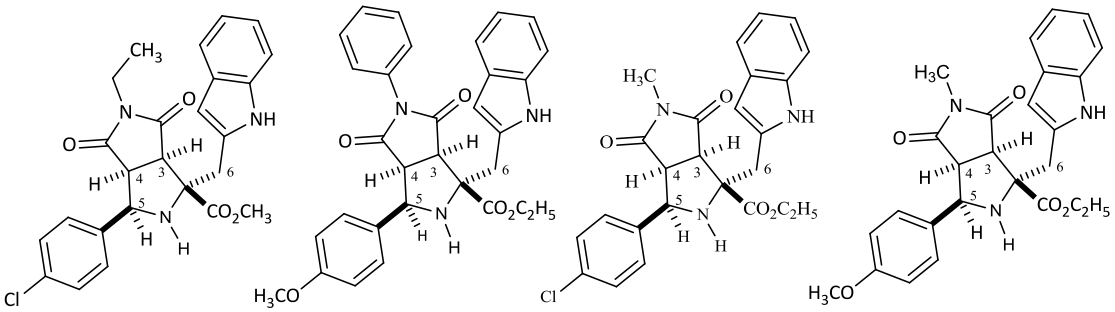


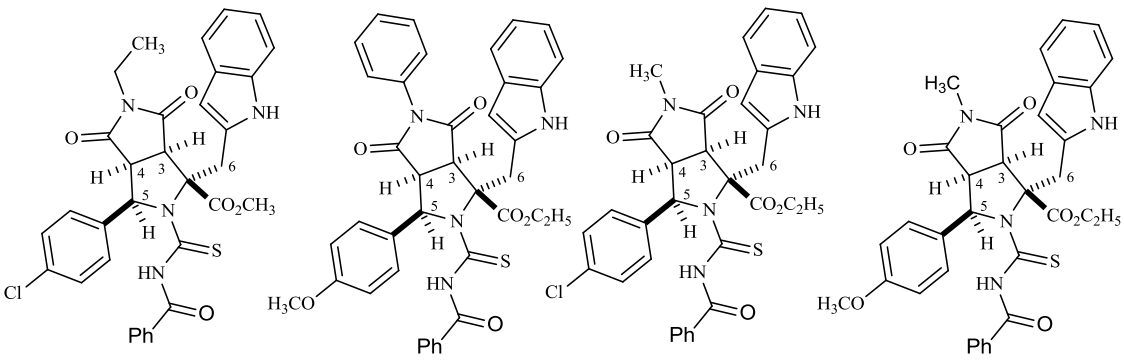
Figure 6. MM2 minimal energy calculations [36] of compounds **7b** and **8**.

Scheme 1



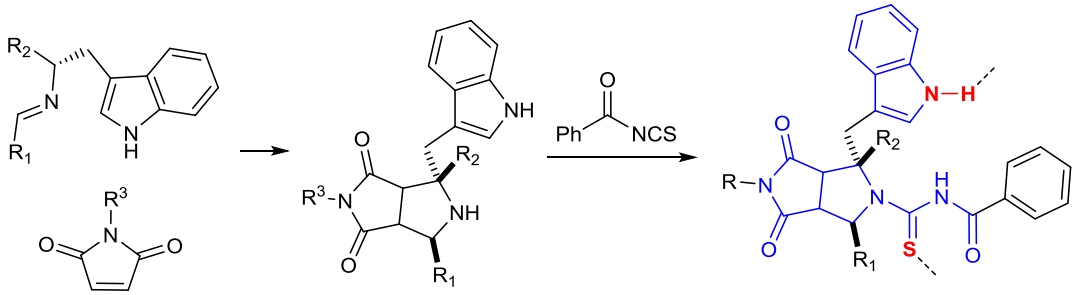


58% 6a 87% 6b 76% 6c 75% 6d



73% 7a 90% 7b 78% 7c 90% 7d

Graphical Abstract



BIOACTIVE MOLECULE